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*ELECTRICAL CORRELATES OF PURE AND HYBRID STRAINS OF SWEET CORN**

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It has been said by numerous investigators that the key problem in biology is organization. The patterns which living organisms present form the basis of description and classification in natural history. Adequate as these are in many instances, little evidence has been forthcoming concerning the forces which impose this stable and characteristic pattern on the dynamic flux which is unique in living systems. The geneticists have been extraordinarily successful in showing that many of the properties of the pattern can be related to structural elements in the chromosomes, including even molecular linkages. The mechanism by means of which the genes control form is still hidden. It is at this point that the key problem, the pattern of organization, arises.

The search for an answer to the problem has fallen rather naturally into three channels. In the first, the investigations have tended to assume that the forces operating were peculiar to biological systems. This has led to a number of fruitful qualitative descriptions such as physiological gradient, biological and embryological fields. These terms include no quantitative measures and, therefore, cannot be genuinely rigorous. The most popular approach has been through chemistry. The relationship between morphogenesis and chemical entities and processes has been widely examined. Out of such studies has come a series of descriptions of the building blocks of which protoplasm is composed, but so far it has failed to provide significant evidence of how, in the midst of constant chemical change, the design remains so astonishingly constant. The third channel is still relatively unexplored. A few sporadic and still unsuccessful attempts have been made to influence the shape of living things through physical force. However, the development of modern techniques of electrical measurement has made it possible to explore the electrical correlates of this design more effectively.

In 1932 Burr,[1] and in 1935 Burr and Northrop,[2] proposed a working hypothesis to the effect that the electrical signs to be found everywhere among living things indicate the existence of an underlying electrodynamic field whose characteristic forces impose pattern on protoplasm. In the past seven or eight years, a number of studies coming from Burr's laboratory have tended to support the validity of the working hypothesis. More particularly, in 1941[3] he showed that in the developing frog's egg a characteristic electrical pattern could be determined in the unfertilized egg, and in the fertilized egg before the advent of the characteristic form of the embryo. The axis of this electrical pattern was found to predict the longitudinal axis of the nervous system. These findings suggest that there is a close relationship between the electrical pattern and the bilateral symmetry of the embryo. Genetics has shown the structural pattern to be closely associated with chromosomal arrangement. It follows, therefore, that in all probability, there is a very close relationship between electrical pattern and the genetic constitution of the living organism. To investigate this problem is difficult in the animal kingdom. In the plant world, however, the situation is simpler for genetic controls are more readily imposed and the consequences more readily observed.

With the coöperation of the authorities of the Connecticut Agricultural Experiment Station, it has been possible to study the electrical patterns in several pure and hybrid strains of sweet corn. The corn seeds used were from strains which have been under study for some time. These strains differ considerably in genetic constitution and in the degree of hybrid vigor shown in crosses between them.

The seeds were collected in 1942 and studied during the winter of 1942–1943. Four inbred strains were studied, and three hybrids. The inbreds used were *C*6, *C*13, *P*39, and a mutant of *P*39, *C*30. The hybrids were *P*39 × *C*13, *C*30 × *C*13, and *C*6 × *C*13. *C*6 is the seed parent of Whipcross *C*6.2, with ears set low on stalk and well formed. It is resistant to bacterial wilt and rust. *C*13 is a Golden Early Market inbred. It is used as the seed or parent pollen of Marcross, *C*13.6. It is highly resistant to bacterial wilt. *P*39 is a mid-season yellow sweet corn, inbred, of unknown origin. *C*30 is a semi-dwarf mutant of *P*39. It is normal in appearance though much reduced in size. It is completely recessive to the *P*39. It has been shown by Singleton[4] to differ by only a single gene from *P*39.

The hybrids studied have shown considerable differences in hybrid vigor in the field. When *P*39 is crossed with *C*13, the average weight of the ears is significantly less than in the hybrid *C*30 × *C*13. This was true in 1940 and again in 1942. In 1941, however, there was no difference. In spite of the dwarfism of *C*30, the hybrids from this line seem to have slightly more hybrid vigor than *P*39 hybrids. The hybrid *C*6 × *C*13, known as "Marcross," is still the outstanding early season hybrid (Singleton, personal com-

munication). In this material, therefore, there are available four stable pure strains of significantly different properties with which to correlate electrical patterns. The three hybrids between them show gradation of hybrid vigor which is least in *P*39 × *C*13 and greatest in *C*6 × *C*13. If electrical patterns have any significance, electrical correlates of these differences should be manifest.

The technique employed was standard, consisting of the determination of a standing potential between the two opposite ends of the longitudinal axis of the kernel. The point of the attachment of the kernel to the cob was invariably positive and the opposite pole negative. Contact with the system was made by silver-silver chloride electrodes and measurements made with a microvoltmeter and galvanometer. More than 2000 measurements were made under carefully controlled conditions with the results shown in table 1.

A statistical analysis† shows that the *F* factor is 6.4. The requirement for 1% significance is 3.07. It is safe to conclude, therefore, that the means of all the strains differ significantly from each other. A second set of determinations was carried out using a larger number of kernels but with a smaller number of measurements for each kernel, and the means were found to check closely with those shown in table 1. Aside from the generally different means the most striking finding was the very great difference between the mean of the single gene mutant *C*30 and the parent stock *P*39. It is remarkable that the change of a single gene in *P*39 should produce such a profound and significant change in the over-all pattern of voltage difference.

TABLE 1

STATISTICAL ANALYSIS OF POTENTIAL DIFFERENCES IN SWEET CORN SEEDS

Means in millivolts

PURE STRAINS				HYBRIDS		
*C*30	*C*13	*C*6	*P*39	*P*39 × *C*13	*C*30 × *C*13	*C*6 × *C*13
6.2	19.4	23.8	24.05	14.5	17.4	23.3

$F = 6.4$. 1% Sig., 3.07. 5% Sig., 2.23.

The conclusion seems to be inescapable, that there is a very close relationship between the genetic constitution and the electrical pattern. If further studies should confirm this conclusion, it seems very probable that one of the ways the chromosomes impart design to protoplasm is through the medium of an electrodynamic field.

No less interesting than the electrical correlates of the pure strains is the relationship between the potential differences and hybrid vigor. The electrical studies show a significant relationship between the potential difference and the degree of hybrid vigor, a relatively high potential difference

being found in association with a high degree of hybrid vigor in the field; and a lower difference with a lower degree of vigor.

The search for a significant measure of hybrid vigor in plants has yielded confusing and inconclusive evidence. The findings here reported suggest that by means of the measured potential difference it may be possible to predict hybrid vigor.

Summary.—1. Electrical correlates have been found between different inbred strains of sweet corn even when the difference is due only to a single gene.

2. The magnitude of the potential difference is positively correlated with the degree of hybrid vigor.

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† The aid of Dr. Chester I. Bliss and Professor I. L. Child is gratefully acknowledged.

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SYNTHESIS OF RIBOFLAVIN BY A YEAST

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Previous investigations have shown that yeasts require one or more members of the vitamin B complex in order to grow well in chemically defined media. It appears, however, that riboflavin (vitamin B_2) is not needed as a constituent of the culture media supporting yeast growth. Since riboflavin is known to play an important rôle as a respiratory enzyme generally throughout the plant and animal kingdoms, it seems probable that yeasts are able to synthesize this vitamin in amounts sufficient at least for their own requirements. In a preliminary survey of the production of vitamin B_2 by some 200 kinds of yeasts, one species has been found to produce extraordinary amounts of riboflavin in synthetic culture media.* This yeast offers, therefore, certain obvious advantages for studies on vitamin synthesis, and assumes special significance in relation to the commercial production of vitamin B_2 for use in animal and human nutrition. A brief account will be given concerning the influence of various factors, such as temperature, aeration, cyanide, composition of the medium, etc., upon the yield of riboflavin. The results of other experiments, including the

feeding of the yeast to animals for the purpose of determining possible toxicity, will be published elsewhere.

The yeast which has been employed for most of the work reported herewith was received as an unidentified species number 488 from Dr. Lynferd J. Wickerham, who isolated it from sour milk some six years ago in the Yale laboratories. Additional strains of yeasts which appear to belong in the same species were isolated from various sources and supplied to us by Dr. Wickerham. These organisms were found to produce riboflavin in varying amounts, indicating that appreciable biochemical variation exists among different strains of the yeast.

When the yeast was cultivated in mineral salts-glucose solution enriched with asparagine and biotin, the resulting fermented liquor possessed a deep yellow color not unlike that of synthetic riboflavin solutions. Upon irradiation with ultra-violet, the fermented liquor exhibited a green fluorescence. Further fluorometric determinations performed upon the eluate from florisil, and microbiological assays made on the cultures with the test organism *Lactobacillus casei* indicated the presence of large amounts of riboflavin in the fermented liquor. Separation of the yeast from the fermented medium by means of filtration or centrifugation yielded a clear yellow solution, and a mass of almost white yeast cells. Microbiologcal assays further indicated that nearly all the vitamin B_2 accumulates outside the yeast in the fermented liquor, thus eliminating the necessity for extracting the vitamin from the yeast by means of expensive solvents. Although riboflavin was produced by all strains of the species tested, strain designated No. 488 was found to be particularly efficient.

Composition of the Medium.—The culture solution employed in this study, unless stated otherwise, had the following composition per liter: KH_2PO_4, 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $CaCl_2 \cdot 2H_2O$, 0.3 g.; $(NH_4)_2SO_4$, 2.0 g.; KI, 0.1 mg.; dextrose, 20 g.; asparagine, 2.0 g.; biotin methyl ester, 1.0 μg. Trace elements were added in p. p. m. as follows: B, 0.01; Mn, 0.01; Zn, 0.07; Cu, 0.01; Mo, 0.01; and Fe, 0.05. Distilled water was employed in making the medium. The reaction of the culture solution was adjusted to pH 5.0. The medium was dispensed usually in the amount of 25 ml. into each 125-ml. Erlenmeyer flask. The flasks were plugged with cotton and sterilized by autoclaving at 120°C. for 15 minutes. After cooling, inoculation was accomplished by adding a drop of yeast suspension to each flask.

Variations in or omission of certain constituents of the medium, and the influence of substituting or adding other compounds were studied in relation to growth and the production of riboflavin. After incubating usually at 30°C. for about one week, growth of the yeast cultures was measured turbidimetrically, with appropriate corrections being made for the colored solutions. Since the amounts of the vitamin produced were relatively

great, it was found convenient to make quantitative determinations by absorption photometry. The yeast was removed from the cultures by filtration through supercel in a Büchner funnel. Corrections for absorption resulting from the presence of pigments other than riboflavin in the medium were made with the aid of uninoculated controls. Absorption of blue light by the solutions was determined with a Klett photoelectric colorimeter and the resulting values were then calculated in terms of vitamin B_2 by reference to a standard curve constructed for solutions of synthetic riboflavin. Microbiological assays gave good agreement with the photometric determinations.

The Source of Carbon.—A very important factor in the production of riboflavin is the source of carbon for fermentation. It appears that certain kinds of carbon compounds supplied in the medium may be used for the building of protoplasm but remain unavailable for synthesis of riboflavin. Certain sugars, such as Pfanstiehl's C.P. maltose and *d*-galactose, produce excellent growth, but yield only small amounts of riboflavin. Sucrose, dextrose and levulose promote both growth and production of vitamin B_2. When *d*-galactose was mixed with dextrose in the medium, excellent growth resulted, but only low yields of riboflavin were observed.

The kind of hexose sugar as well as its concentration in the medium appear to have a direct influence upon the production of pentose for incorporation into the riboflavin molecule. The influence of different concentrations of dextrose upon growth and yield of vitamin B_2 is shown graphically in figure 1. The experimental results presented in figure 1 were obtained with glycine and asparagine each present in the amount of 0.5 g. per liter of basal medium. Growth was allowed to proceed at 30°C. for a period of six days.

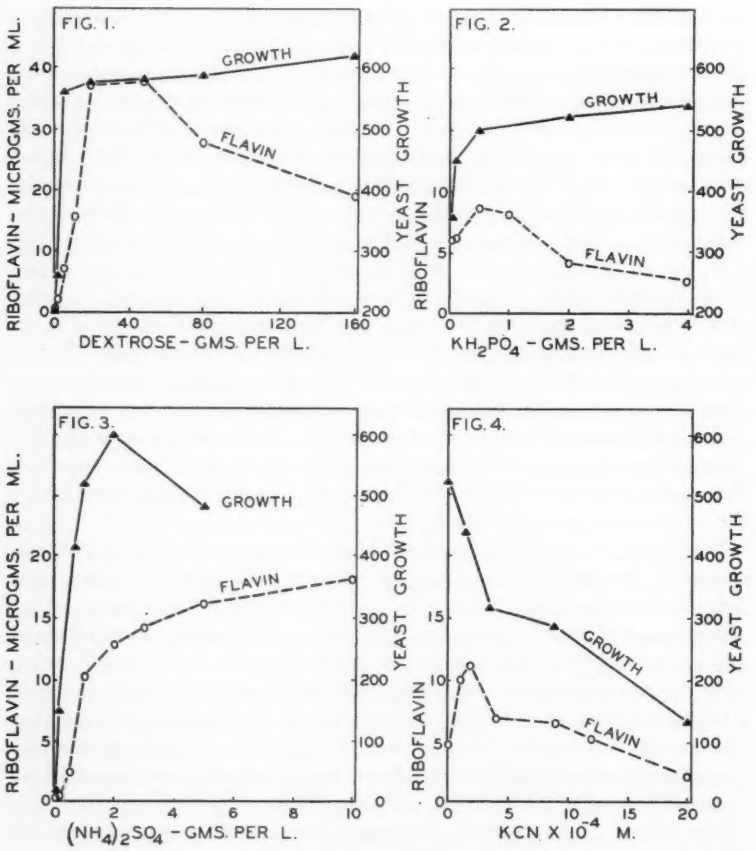
The Source of Nitrogen.—A suitable source of organic nitrogen seems to be of considerable importance for the formation of vitamin B_2. Appreciable amounts of the vitamin accumulated during fermentation of mineral-dextrose solutions containing nitrogen supplied only in inorganic form as $(NH_4)_2SO_4$. The addition of certain amino acids such as glycine, or amides such as asparagine served to augment the yield of riboflavin, without necessarily affecting growth of the yeast. Asparagine appeared to be more efficient than glycine for the production of vitamin B_2. A favorable medium may contain 0.5 g. of glycine and 0.5 g. of asparagine in addition to the usual 2.0 g. of $(NH_4)_2SO_4$ in 1 liter of the basal medium. Whereas, the yield of riboflavin varied with the increase of glycine and asparagine over a considerable range, production of the vitamin was decreased by adding hydrolyzed casein in amounts from 0.5 g. to 4.0 g. per liter. The growth obtained with supplements of casein, however, was at least equal to that observed in media containing glycine and asparagine. Other amino acids supplied in addition to asparagine may have little effect

upon yield, or in some instances may greatly enhance the production of riboflavin. When amino acids were added singly at the rate of 0.8 g. per liter along with asparagine occurring as 2.0 g. per liter in the medium the effectiveness for increasing the yield was as follows in ascending series: serine < aspartic acid < arginine < leucine <valine <glycine < methionine. Under comparable conditions the production of riboflavin in micrograms per ml. of fermented liquor was as follows: a medium containing N only as $(NH_4)_2SO_4$ in the amount of 2.0 g. per liter yielded 12.5 γ B_2 per ml.; with asparagine added in the amount of 2.0 g. per liter, 18.0 γ B_2 per ml. were produced; with asparagine 2.0 g. per liter plus glycine 0.8 g. per liter, 47.0 γ B_2 per ml.; and with asparagine 2.0 g. per liter plus methionine 0.8 g. per liter, 60.0 γ B_2 per liter were observed. It is of interest to point out that both asparagine and glycine are relatively efficient and comparatively inexpensive sources of organic nitrogen. In the experiment where 2.0 g. of asparagine and 0.8 g. of glycine per liter were employed, and fermentation allowed to proceed at 30°C. for about six days, the fermented liquor was separated from the yeast cells by filtration. Dehydration of the fermented liquor yielded a yellow powder which contained 7 mg. of riboflavin per gram of solids.

Inorganic Compounds.—Variations in the amounts of inorganic materials present in the medium were found to influence both growth and riboflavin content of the fermented liquor. In one experimental series using 50 ml. of medium and 2.0 g. of asparagine per liter and a growing temperature of 27°C., the supply of KH_2PO_4 in the nutrient solution was varied over a wide range. As shown in figure 2, riboflavin reached a maximum with KH_2PO_4 supplied at levels of about 0.5 to 1.0 g. per liter, and decreased with greater doses of the salt. Growth of the yeast rose markedly with increased amounts of KH_2PO_4 in the lower range, and was maintained for all values of the salt employed up to 4.0 g. per liter.

The influence of increasing the supply of $(NH_4)_2SO_4$ upon growth and yield of riboflavin in 50-ml. cultures growing at 27°C. is shown in figure 3. Growth increased sharply with increments of ammonium salt up to about 2.0 g. per liter. Above this level some decrease in growth was apparent. The formation of riboflavin in the fermented liquor was augmented greatly by increasing $(NH_4)_2SO_4$ up to about 1.0 g. per liter, and was somewhat enhanced over the remainder of the range up to 10.0 g. per liter.

Influence of Cyanide.—Inasmuch as cyanide is known to inhibit the cytochrome system of respiration but not the riboflavin mechanism, it seemed desirable to test the effect of cyanide upon the formation of riboflavin by the yeast. The effects of cyanide upon growth and riboflavin production were studied in two experiments. Cyanide stock solution was prepared by dissolving KCN in distilled water. This solution was sterilized by filtration through a Berkfeld filter and small measured volumes were then



FIGURES 1–4

Growth and production of riboflavin by a yeast in relation to chemical compounds present in the nutrient medium. (1) Sugar is required for growth and synthesis of riboflavin, and amounts between 20 and 50 g. per liter appear to be near the optimum. (2) Potassium phosphate supplied from 0.5 to 1.0 g. per liter is near optimum; further increase of the salt stimulates growth somewhat but decreases the production of riboflavin. (3) With an increasing supply of ammonium sulphate as the sole source of nitrogen, both growth and synthesis of riboflavin are augmented. (4) Addition of small amounts of cyanide to growing cultures stimulates the synthesis of riboflavin but inhibits growth of the yeast.

added to the autoclaved and cooled basal culture medium to produce concentrations of KCN of 10^{-3} and 10^{-4} molar. Following inoculation of the cyanide treated media, growth and riboflavin formation were inhibited for a

period of several days, but after about a week the yeast began to grow and produce good yields of riboflavin.

In another experimental series kept at 25°C. potassium cyanide was added aseptically in different amounts to 50-ml. cultures which had been growing for about 24 hours. The production of the vitamin was increased appreciably by the presence of cyanide in the range of 1 to 4 $\times$ 10^{-4} molar, as indicated in figure 4. Growth of the yeast, however, was hindered by cyanide at all dosage levels. The addition of small amounts of cyanide to growing cultures would seem to offer a method for speeding the production of vitamin B_2. Although the mechanism by which cyanide increases the formation of riboflavin is not known, the phenomenon is of considerable interest in view of the well-known action of cyanide in poisoning the cytochrome system in aerobic organisms.

Effect of Aeration and Temperature.—In order to determine the influence of oxygen upon growth and yield of riboflavin, cultures were set up at 27°C. in air and in nitrogen contained in an anaerobic jar. At the end of six days, growth of the yeast was about the same in the two situations, but the production of riboflavin was reduced to a very low level in nitrogen. In another experiment performed with filtered air bubbling continuously through the medium, the yield of riboflavin was diminished as compared with the amounts formed by cultures kept in still air. When 50 ml. of medium were employed instead of 25 ml. in a 125-ml. flask, the production of riboflavin was diminished, perhaps because of suboptimum supply of oxygen.

For the purpose of ascertaining the effect of temperature upon growth and yield of riboflavin, a series of 50-ml. cultures were allowed to develop at 20°, 27°, 30° and 36°C. for one week. Multiplication of the yeast was rapid in the range 27° to 30°C., and the yield of the vitamin greatest at 30°C.

Summary.—Certain strains grown for a period of four to six days in synthetic culture media have been found to produce from 10 to 60 micrograms of riboflavin (vitamin B_2) per ml. of fermented liquor, depending upon environmental conditions. High yields of the vitamin are obtained by supplying certain organic nitrogenous compounds such as asparagine, glycine and methionine in the medium. The addition of hydrolyzed casein permits excellent growth, but inhibits formation of the vitamin. Sucrose, dextrose or levulose serve as suitable sources of carbon for growth and production of riboflavin. Synthesis of the vitamin is inhibited by the presence of maltose or galactose, though growth is not diminished by these sugars. Appropriate concentrations of salts, a temperature of about 30°C. and suitable aeration are important for obtaining efficient yields of the vitamin. Under anaerobic conditions the formation of riboflavin is inhibited without any decrease in growth. Small amounts of cyanide added aseptically to growing cul-

tures stimulated production of the vitamin but inhibited growth. It is apparent that synthesis of protoplasm and the production of riboflavin are related in different ways to the utilization of various compounds present in the culture medium. The ability of this yeast to synthesize extraordinary amounts of riboflavin appears to be genetically determined, but the extent to which this synthetic capacity is expressed depends a great deal upon cultural conditions.

* For obvious reasons it seems best not to publish the specific name of this yeast at present.

PSEUDOPYRIDOXINE AND CERTAIN FUNGI

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Snell, Guirard and Williams,[1] Snell[2] and Snell and Guirard[3] have reported a series of observations on the relation of *Streptococcus lactis* R to pyridoxine from which the following conclusions, among others, may be drawn.

1. Pyridoxine as such is inactive.
2. An active compound, pseudopyridoxine, is formed when pyridoxine is autoclaved with amino acids.
3. Pyridoxine may be completely replaced in its growth effect by sufficient amounts of *dl*-alanine.

We have been interested in determining whether these conclusions apply to certain filamentous fungi which are known to be pyridoxine-deficient.[4]

The organisms used were *Ophiostoma catonianum*, *Ceratostomella ips* No. 255, *C. microspora*, *C. montium*, *C. multiannulata*, *C. piliferum*, *C. pluriannulata* and *C. ulmi*. Each fungus was grown in duplicate at 20°C. in 25 ml. of a basal mineral-dextrose medium containing asparagine and supplemented with biotin and thiamine.[5] Observations on growth were made at intervals and after from 9 to 21 days, depending upon the rapidity of growth of the fungus, dry weights were determined by filtering into Gooch crucibles and drying at 100°C.

Activity of Pyridoxine.—In our experiments pyridoxine as such was effective for all eight of the fungi investigated. This was demonstrated by the following observations:

Little or no growth occurred in the basal solution. The addition of pyridoxine permitted considerable growth and no consistent significant

differences were noted in the action of pyridoxine sterilized with the constituents of the basal solution or added after the basal solution had been previously autoclaved (table 1). In our basal solution asparagine serves as a source of nitrogen. In order to eliminate the possibility that asparagine and pyridoxine might yield an active substance the asparagine was replaced by an equivalent amount of nitrogen in the form of NH_4NO_3. Again little or no growth was observed without the addition of pyridoxine to the medium, and the activity of the pyridoxine was not increased by sterilizing it with the nutrient solution as compared to adding it after the basal medium had been autoclaved (table 1).

TABLE 1

DRY WEIGHTS IN MG. PER CULTURE OF FOUR FUNGI IN A BASAL SOLUTION SUPPLEMENTED WITH PYRIDOXINE, CASEIN HYDROLYSATE AND *l*-CYSTINE HCl

ADDITIONS TO 25 ML. OF BASAL SOLUTION	*Ophiostoma catonianum*		*C. pluriannulata*		*C. montium*		*C. microspora*	
		PYRIDOXINE ADDED AFTER AUTOCLAVING		PYRIDOXINE ADDED AFTER AUTOCLAVING		PYRIDOXINE ADDED AFTER AUTOCLAVING		PYRIDOXINE ADDED AFTER AUTOCLAVING
1 mμ mole pyridoxine	25.2	23.3	22.2	28.8	12.4	14.8	8.9	9.1
20 mg. casein hydrolysate, 0.5 mg. *l*-cystine, 1 mμ mole pyridoxine	27.4	27.1	34.8	48.5	3.8	4.9	9.5	9.1
20 mg. casein, hydrolysate, 0.5 mg. *l*-cystine	0.8	..	1.0	..	0.1	..	0.2	..
Asparagine replaced by 0.5 mg. NH_4NO_3, 1 mμ mole pyridoxine	18.6	19.2	19.8	21.6	10.9	11.3	5.9	8.4
None	0.3	..	0.2	..	0.1	..	0.1	..

Evidence for Pseudopyridoxine.—No evidence was obtained for the existence of a more active form of pyridoxine. Growth in the basal medium plus 1, 5 or 10 mg. of *dl*-alanine, and 1 mμ mole of pyridoxine per flask was approximately the same whether the alanine was sterilized with the pyridoxine in the nutrient solution or added under sterile conditions after the balance of the solution had been autoclaved (table 2). Growth in the basal medium plus 20 mg. of casein hydrolysate,[6] 0.5 mg. of *l*-cystine HCl and 1 mμ mole of pyridoxine per flask was the same whether the pyridoxine was sterilized along with the other constituents of the nutrient solution or added after the balance of the solution had been autoclaved (table 1).

We expected that the response of the fungi might be greater in the medium containing pseudopyridoxine, i.e., the one in which the amino acids and pyridoxine were autoclaved together. No evidence for this effect was obtained.

Substitution of dl-Alanine for Pyridoxine.—We were unsuccessful in attempts to substitute *dl*-alanine for pyridoxine. Growth was not improved by the addition to the basal solution of 1, 5 or 10 mg. of alanine per flask. The same result was obtained when the alanine was autoclaved in the nutrient solution, and when it was added after the balance of the solution had been autoclaved (table 2). Since growth was obtained when pyridoxine was added to the solutions containing alanine, it appears that the lack of growth in the presence of alanine and absence of pyridoxine was not because of a toxicity of alanine, but because that substance did not replace pyridoxine for these fungi.

TABLE 2

DRY WEIGHTS IN MG. PER CULTURE OF FOUR FUNGI IN A BASAL SOLUTION SUPPLEMENTED WITH *dl*-ALANINE AND PYRIDOXINE

ADDITIONS TO 25 ML. OF BASAL SOLUTION	*Ophiostoma catonianum*		*C. pluriannulata*		*C. montium*		*C. microspora*	
		ALANINE ADDED AFTER AUTOCLAVING		ALANINE ADDED AFTER AUTOCLAVING		ALANINE ADDED AFTER AUTOCLAVING		ALANINE ADDED AFTER AUTOCLAVING
10 mg. alanine	0.5	0.2	0.3	0.6	0.5	0.4	0.8	0.3
5 mg. alanine	0.6	0.5	0.3	0.2	0.4	0.7	0.7	0.2
1 mg. alanine	0.4	0.1	0.2	0.3	0.5	0.4	0.7	0.4
10 mg. alanine, 1 mμ mole pyridoxine	29.2	31.3	26.3	29.6	25.7	21.6	14.4	14.4
5 mg. alanine, 1 mμ mole pyridoxine	29.9	26.5	32.3	24.6	25.3	24.0	12.9	14.1
1 mg. alanine, 1 mμ mole pyridoxine	31.9	26.8	36.1	28.0	24.5	22.7	13.6	13.5
1 mμ mole, pyridoxine	31.5	..	25.6	..	20.9	..	15.1	..
None	0.4	..	0.3	..	0.4	..	0.3	..

Discussion.—From our experiments it appears that the eight fungi we used differ from *S. lactis* R in their response to pyridoxine and to *dl*-alanine. Snell and Guirard[3] state that for *Lactobacillus casei* also alanine does not replace pyridoxine, but that *S. lactis* 125 responds as does *S. lactis* R.

If we assume in accordance with Snell and his associates that

$$dl\text{-alanine} \rightarrow \text{pyridoxine}$$
$$\text{pyridoxine} + AA \rightarrow \text{pseudopyridoxine}$$

and that pseudopyridoxine is the active substance, then the following conclusions and assumptions would follow:

The eight fungi we investigated are unable to transform *dl*-alanine to pyridoxine under the conditions of our experiments.

These fungi produce adequate amounts of *AA* from sugar and inorganic nitrogen, but are unable to synthesize pyridoxine. When furnished pyridoxine they synthesize *AA* and combine the two intermediates (pyridoxine and *AA*) into pseudopyridoxine. This situation would be analogous to that which exists in the relation of the fungus *Ceratostomella montium* and numerous others to thiamine and its intermediates. *C. montium* is able to synthesize the thiazole portion of the thiamine molecule from the elementary constituents of a basal medium, but cannot make the pyrimidine intermediate. Furnished pyrimidine this fungus constructs thiazole and combines the two substances into the essential thiamine.[6]

On the basis of the assumptions made above we might expect to find organisms with the following deficiencies for pseudopyridoxine and its immediate precursors.[7]

1. Those requiring for growth the presence in the medium of pseudopyridoxine as such. This group would include organisms unable to synthesize pseudopyridoxine from its intermediates. *S. lactis* R and *S. lactis* 125 would probably belong in this group.

2. Organisms able to grow if supplied with both pyridoxine and the *AA* factor, but unable to grow with either one alone. These would be unable to construct either constituent of pseudopyridoxine, but would be capable of making the active pseudopyridoxine if furnished its intermediates. Animals would belong to this group or the next.

3. Organisms able to grow with pyridoxine present in the medium. This group would be able to synthesize *AA* but not pyridoxine. Supplied with pyridoxine they could construct *AA* and combine the two intermediates to form pseudopyridoxine. The eight filamentous fungi we used, and perhaps *Lactobacillus casei* also, would belong to this group.

4. Organisms able to grow with *AA* but no pyridoxine in the medium. This group would include those able to synthesize pyridoxine but unable to make the *AA* factor.

The validity of the suggested classification of organisms in relation to pseudopyridoxine depends, of course, upon further information on the existence, origin and composition of pseudopyridoxine and upon additional observations on its significance for various organisms.

Summary.—Eight filamentous fungi were found to respond to pyridoxine as such. The physiological activity of pyridoxine was not replaced by *dl*-alanine. No evidence was obtained from these fungi for the existence of a more active form of pyridoxine, Snell's pseudopyridoxine.

[1] Snell, E. E., Guirard, B. M., and Williams, R. J., *Jour. Biol. Chem.*, **143**, 519–530 (1942).

[2] Snell, E. E., *Proc. Soc. Exp. Biol. Med.*, **51**, 356–358 (1942).

[3] Snell, E. E., and Guirard, B. M., *Proc. Nat. Acad. Sci.*, **29**, 66–73 (1943).

[4] Robbins, W. J., and Ma, R., *Bull. Torrey Club*, **69**, 184–203 (1942). Robbins, W. J., and Ma, R., *Arch. Biochem.*, **1**, 219–229 (1942). Robbins, W. J., and Ma, R., *Am. Jour. Bot.*, **29**, 835–843 (1942).

[5] The basal solution contained per liter 50 g. dextrose, 1.5 g. KH_2PO_4, 0.5 g. $MgSO_4 \cdot 7H_2O$ and 2 g. asparagine. To this solution the following trace elements were added in p. p. m.: 0.005 B, 0.02 Cu, 0.1 Fe, 0.01 Ga, 0.01 Mn, 0.01 Mo and 0.09 Zn. To each flask there were added also 0.1 μg. biotin methyl ester and 10 mμ moles thiamine. For the experiment summarized in part in table 1 the reaction of the basal solution was adjusted with KOH to pH 5.2 and for the one presented in table 2 to pH 5.8. Solutions were autoclaved at 15 lbs. pressure for 30 minutes.

[6] We used S. M. A. vitamin-free casein hydrolysate which had been treated with excess $CaCO_3$ to neutralize the acidity.

[7] Robbins, W. J., and Ma, R., *Bull. Torrey Club*, **70**, 190–197 (1943).

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*THE DISTRIBUTION OF PHOSPHATASE IN THE SPINAL CORD OF CHICK EMBRYOS OF ONE TO EIGHT DAYS' INCUBATION**

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Despite the enormous interest that has grown around the phosphatases since their discovery 36 years ago, our knowledge of the rôles they play in the economy of living organisms, in which they are almost ubiquitous, is but slight. In part the reason for this lack is, as Kay (1932) pointed out in another connection, the fact that the method of studying phosphatase activity in tissue extracts precludes any chance of discovering the intimate distribution of the enzyme within the tissues. In 1939, however, Gomori, and also Takamatsu, offered a histochemical method whereby the localization of alkaline phosphatase in cells and tissues may be determined with great exactness, and more recently Gomori (1941*b*) has extended this method to acid phosphatase.

With the thought that a histochemical study of the embryogenesis of these enzymes might produce clues concerning their biological significance, the writer undertook a comprehensive survey of the development and distribution of phosphatases in the embryo of the chick. Among the striking results to emerge from this study is the fact that both these enzymes appear in a remarkable pattern in the embryonic spinal cord.

Method.—The techniques outlined by Gomori (1941*a*, *b*) for the histological demonstration of alkaline and acid phosphatases were followed.

They were used on White Leghorn embryos of one to eight days' incubation, which were fixed in ice-cold acetone for 24 hours, embedded in a paraffin-beeswax-bayberry wax mixture by routine methods, and sectioned serially at 15 micra. Chilled acetone proved an excellent fixative for the gross histological examination of objects even as large as eight-day chick embryos, but the cell structures were somewhat distorted by shrinkage.

The sodium glycerophosphate used contained equal parts of the alpha and beta salts; pure alpha glycerophosphate was not available. The incubating solution for alkaline phosphatase was made according to Gomori's (1941*a*) instructions, at pH 9.3. Incubation was continued for three hours at 38 degrees. After treatment with cobalt nitrate and ammonium sulfide, the sections were counter-stained lightly with erythrosin. To compensate for the precipitation of lead beta-glycerophosphate, the formula (Gomori, 1941*b*) for the incubating solution for acid phosphatase, was altered as follows:

Molar acetate buffer at pH 4.8	$2^1/_2$ parts
5% $PbNO_3$	2 parts
Distilled water	30 parts
Na glycerophosphate	5 parts

The solution was made up fresh for each test, and the precipitate removed by filtration. The pH is 5.1. Incubation was continued for six hours at 38 degrees. The sections were then treated with sulfide and counter-stained as before. Collodion protection was not found useful in either case.

The alkaline phosphatase showed remarkable stability in the course of handling. Keeping the specimens in acetone in the refrigerator for a week did not cause any variation in the excellently uniform pictures, nor did allowing the paraffin blocks or even the unstained mounted sections to stand for a month have any deleterious effect. The acid phosphatase, on the other hand, proved extremely labile, even on standing at icebox temperature, so that the achievement of comparable results required that the embryos be processed as rapidly as possible; three to four days were generally allowed from killing to staining. The embedding heat also had to be controlled carefully for, unlike the enzyme reported by Gomori (1941*b*) for adult mammalian tissues, this phosphatase was destroyed by temperatures above 60 degrees. Yet, in spite of these precautions, individual embryos showed considerable variation in the intensity of acid phosphatase activity. The pattern of distribution in the various tissues, however, was constant.

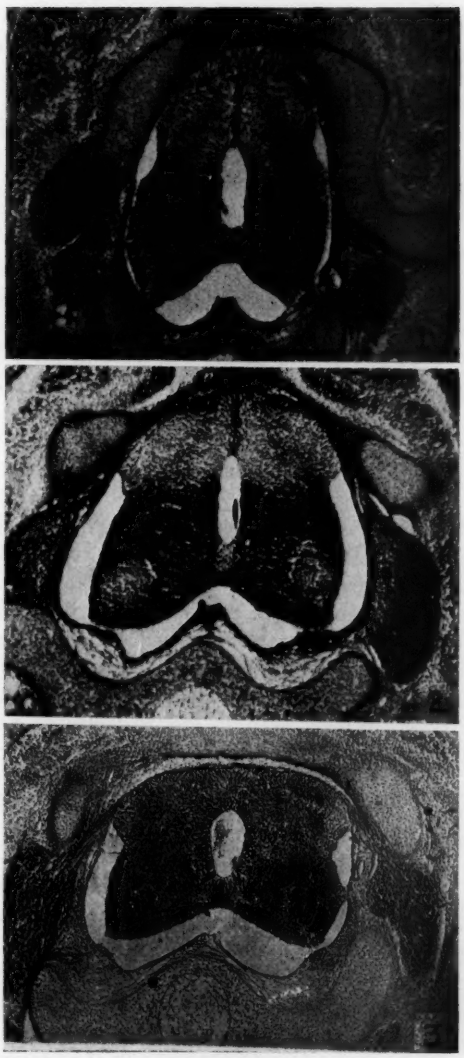
Results.—(1) *Alkaline Phosphatase.* The activity of alkaline phosphatase, as judged by the amount of phosphate deposited within a given time,

is generally strong in the embryo, for heavy black deposits appear in many tissues even if incubation is continued for only one hour. A high concentration of alkaline phosphatase is found in the neural tissue at the end of the first day, the earliest stage examined. Through the second day the neural tube is uniformly active at anterior levels, but near the end of the region of differentiated somites the reactivity wanes and almost disappears. Anteriorly, on the third day, the dorsal half of the cord and the neural crests tend to react more strongly than the ventral half, but this difference fades into uniformity at posterior levels. On the next two days small portions of the ventral region of the cord continue to lose their alkaline phosphatase content, and it soon becomes clear that these portions represent the future motor horns of the limb plexus. The white matter differentiating outside of the horn area is strongly positive. In the tail region, on the fourth day, the distribution is still fairly uniform.

From the end of the fourth day on, the enzyme disappears according to an antero-posterior gradient from the dorsal half of the gray matter, while it becomes more concentrated in the ventral half, so that by the seventh day a definite and apparently persistent pattern is established. The striking feature of this pattern is a heavy black crossband that virtually bisects the cord; the band reaches from the lumen to the outer edge, thus involving ependymal, marginal and mantle layers. Dorsal to the band the ependyma and gray matter are almost negative, except for a small positive area just above the lumen, at the base of the dorsal funiculus; but ventrally both layers show a marked reaction, with the exception of a limited portion of the ependyma immediately under the lumen, and of the large lateral motor groups which, by the sixth day, are established in the pelvic as well as the pectoral region. The mesial motor groups are also less active than the surrounding gray matter, but the demarcation is not as sharp as in the other cases cited. The white matter contains a high concentration of alkaline phosphatase throughout, though the activity is greater ventral to the crossband than dorsal to it; the ventral commissure is extremely active.

This pattern does not change through the eighth day. Its appearance in the cervical and fore-limb regions is shown in figures 1 and 2.

The absence of alkaline phosphatase described refers only to the cytoplasm of the cell bodies and processes in question. Within such cells the nuclear membranes are clearly outlined by black deposits, and the nucleoli are also stained black. In the cells which show phosphatase in their cytoplasm, when the deposits are light enough to permit examination of the interior of the cell, it can be seen that the nuclear membranes and nucleoli are also strongly reactive. There are no cytoplasmic inclusions seen in either case.



FIGURES 1-3

All sections are from one eight-day embryo. Magnification 50 ×. (1) Alkaline phosphatase, neck region. The heavily stained distal portions of the ganglia are made up of the large cells. (2) Alkaline phosphatase, fore-limb region. (3) Acid phosphatase, hind-limb region. Note the darkly stained masses in the large cells of the ganglia.

In the sensory ganglia, which are made up of cells of two sizes, a clear-cut disparity exists in the alkaline phosphatase content of the cytoplasm of the large and the small cells: the former reacts strongly, whereas the latter seems devoid of the enzyme. Thus the clustering of the large cells distal to the nerve cord gives the phosphatase-stained ganglion the appearance of being divided into two parts (see Fig. 1). As in the cells of the cord, the nucleoli and nuclear membranes of the small cells stain deeply, and in addition a faint darkened network, probably of chromatin, may occasionally be seen inside the nucleus. The interior of the large cells is obscured by the heavy sulfide deposits.

The dorsal and ventral roots are strongly positive, as are the nerve trunks peripheral to the ganglia.

Tests run on six-day embryos at pH 8.5, 8.0 and 7.5 showed that the activity is markedly less at pH 8.5 than at 9.3, and it is abolished at pH 8.0 or lower. Other tests, run for one hour, in which the incubating solution was made 0.001 *M* and 0.01 *M* with respect to $MgCl_2$, revealed some activation at the lower concentration and very marked activation at the higher.

(2) *Acid Phosphatase.* Like its alkaline counterpart, acid phosphatase also appears in the neural tissue at the end of the first day, but it does not seem to be as concentrated, or as active, as the alkaline enzyme. Slides treated by the alkaline technique regularly show dense black deposits, whereas after the acid treatment the deposits in the nerve tissue never have more than a deep golden brown color, even when the incubation is continued for 12 hours. Strict comparison between the two enzymes on this basis is not possible, however, since the acid phosphatase could operate on alpha-glycerophosphate only; and the beta salt is the preferred substrate of the phosphomonoesterases of both Class AI and Class AII (Folley and Kay, 1936). But it is noteworthy that Fleischhacker (1938) found a similarly great disparity between the activity of the two enzymes in mammalian brain tissue, even when beta-glycerophosphate was the substrate for both.

As the cell layers of the cord differentiate, acid phosphatase becomes concentrated chiefly in the ependyma, while the surrounding tissue shows lessened activity. Between the fifth and the seventh day acid phosphatase largely disappears from all three layers of the dorsal half of the cord, except for a few cells just dorsal to the lumen—the same spot that also shows alkaline phosphatase activity. In the ventral half the enzyme occurs in reactive cells scattered through a negative ground substance; but heavier uniform concentrations are found in the lateral and mesial motor groups. These deposits appear in posterior progression, demarcating the lateral horn in the fore-limb region on the fifth day, and in the hind-limb region less than a day later. The ventral commissure also stains more heavily

than its surroundings, and so do an extensive group of ependymal cells below the lumen.

Thus a definite pattern, as shown in figure 3, is laid down by the end of the sixth day. Aside from more complete withdrawal of the enzyme from the dorsal half of the cord, the pattern does not change essentially through the eighth day.

Unlike alkaline phosphatase, the acid enzyme is apparently absent from the nuclei of cells which have unreactive cytoplasm. In the reactive cells the nuclei stain more lightly than the cytoplasm and show no inclusions; but there is in each cell a small, intensely stained body outside the nucleus but closely applied to it. The nature of these bodies is not evident.

The small cells of the ganglia appear to contain only a little acid phosphatase in their nuclei and cytoplasm; they do, however, show the same reactive body on the surface of the nuclei as was found in the active cells of the cord. The large cells contain a very large, intensely reactive mass which is bigger than the nucleus and apparently includes all the cytoplasm of the cell body except for a negative rim continuous with the processes. The clump has a granulated appearance which seems to be caused by darkly staining inclusions. Possibly it represents the tigroid substance shrunken into a rounded lump (see Fig. 3).

The dorsal and ventral roots, and the nerve trunks leading from the ganglia, are lightly positive.

Discussion.—In a consideration of the nature of these enzymes, the thought arises that the alkaline phosphatase, at least, is a phosphomonoesterase component of a lecithinase, a substance which might reasonably be expected to occur in nerve tissue. Comparison of the pH tests reported here with those of King (1931), however, enable the dismissal of this possibility; for King showed that lecithinase has a pH optimun of 7.0–7.4, whereas the alkaline phosphatase of this study did not act at all at pH 8.0 or lower. Similarly it can be seen that neither the acid nor the alkaline phosphatase resembles the 5-nucleotidase found widespread in nerve tissue by Reis (1937). The Reis enzyme is only slightly active against glycerophosphate, but these enzymes are as effective on this substrate as are most other phosphatase-containing tissues of the embryo. Although the 5-nucleotidase is virtually inactive at pH 5, it retained considerable activity at pH 9, but again it fails to correspond to the alkaline enzyme of this study in being unaffected by the presence of magnesium ions.

The enzymes found in the embryonic chick cord do, however, correspond well with the brain phosphatases delineated by Fleischhacker (1938) and Kotkova (1939). The latter demonstrated in the brain of the sparrow and pigeon, as well as of members of other classes, phosphomonoesterases with pH optima of 4.5–5.6, and 8.9–9.6; the second was activated by magnesium, and the first inhibited by fluoride. Fleischhacker obtained somewhat

similar results, except that he claimed the acid phosphatase to be activated by magnesium; but the acid activities he reported were in all cases so low that the differences may be unreliable. Probably all these nerve phosphatases can be classified, according to Folley and Kay's (1936) scheme, as phosphomonoesterases of Classes AI and AII. They may be concerned in the metabolism of the carbohydrate substrates of nerve tissue.

The change in the distribution of the embryonic cord enzymes with time indicates that they may have more than one function. The uniform distribution of the enzymes in the neural tissue of the earliest stages, and their later disappearance from certain regions, and concentration in others, are phenomena shared by practically every organ of the embryo (unpublished results). Thus the observer is led to feel that the phosphatases are essential in the initiation, at least, of differentiation; for every indifferent cell contains phosphatase in its cytoplasm. As differentiation proceeds, the enzymes may disappear, or they may become more concentrated. In the latter case the accumulation of the enzymes in local regions may be regarded as a consequence of differentiation in itself, or in other words a chemical differentiation paralleling the histological differentiation; presumably the enzymes are thereafter concerned in the functioning of the specific cells in which they are localized. In this connection it is suggestive that the establishment of the pattern described, and especially the accumulation of acid phosphatase in the motor groups, precedes by about 24 hours the appearance (at $6^1/_2$ to 7 days) of the first local reflexes (Orr and Windle, 1934). In any case the results suggest for phosphatase a hitherto unsuspected rôle in the actual functioning of nerve elements.

That the localization of acid phosphatase at least is not a transitory embryonic phenomenon is shown by the fact that Fleischhacker (1938), using a crude histochemical method adapted directly from the colorimetric method of Kuttner and Lichtenstein, found similar concentrations in the anterior horn cells of adult rats; he did not mention the pH. Gomori's (1941*a*) failure to find any alkaline phosphatase in mammalian nerve tissue, however, remains to be explained.

Summary.—1. The presence of acid and alkaline phosphatases in the spinal cord of the embryonic chick has been demonstrated by the histochemical techniques developed by Gomori.

2. At all stages examined, alkaline phosphatase reacts much more strongly than acid phosphatase.

3. As early as the end of the first day of incubation, both acid and alkaline phosphatase are found in fairly high concentration in the neural tissue.

4. As development proceeds, the alkaline phosphatase becomes localized in the white matter throughout, and in the gray matter and ependyma of the ventral half of the cord, with the exception of the cells of the motor

groups. It is absent from a small part of the ependyma just below the central canal, and is concentrated in a similar small area just above the canal.

5. The acid phosphatase becomes largely restricted to the ventral half of the cord, and is especially concentrated in the motor groups. It also occurs in small regions just dorsal and ventral to the lumen.

6. The pattern described appears in antero-posterior progression on the fourth, fifth and sixth days.

7. The large cells of the spinal ganglia contain large amounts of both acid and alkaline phosphatase, the small cells very little of either.

8. As far as can be detected, all nucleoli and nuclear membranes are positive for alkaline phosphatase.

9. Acid phosphatase is not found in nuclei, but even where the cytoplasm is not reactive the enzyme is generally present in a small body, of unidentified nature, which is closely applied to the nucleus. In the large ganglionic cells, the cytoplasm is not uniformly reactive, but contains a very large rounded clump which is strongly positive.

10. Evidence is presented which indicates that these enzymes are phosphomonoesterases of Classes AI and AII.

11. It is suggested that both these phosphatases first play a part in early differentiation, and later are involved in the specific functions of the cells in which they become concentrated.

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STUDIES ON THE METHANE FERMENTATION. VI. THE INFLUENCE OF CARBON DIOXIDE CONCENTRATION ON THE RATE OF CARBON DIOXIDE REDUCTION BY MOLECULAR HYDROGEN

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Communicated May 3, 1943

The methane-producing bacterium *Methanobacterium omelianskii* has been shown[2, 3, 4] to form methane by reduction of carbon dioxide, using primary and secondary alcohols and molecular hydrogen* as hydrogen donors. As an extension of earlier studies on this process, it seemed desirable to find out how the concentration of carbon dioxide influences the rate of its reduction. In studying this effect, the reaction

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad (1)$$

has been used because the large gas volume change is advantageous for making rate measurements by a manometric technique.

Methods.—A pure culture of *Mb. omelianskii* was grown in a previously described[2] ethanol-bicarbonate medium which was incubated until 3.0 to 5.5 ml. $N/10$ acetic acid had been formed per 10 cc. The cells from a 500-ml. culture were separated by centrifugation, washed once, and resuspended in 10–15 ml. of a solution containing 0.2 per cent NaCl, 0.2 per cent KCl and 0.03 per cent $Na_2S \cdot 9H_2O$, pH 7. These operations must be carried out so as to minimize exposure of the cells to oxygen. The mixture of chlorides is preferable to distilled water because it increases the rate of sedimentation. The final suspensions contained 0.4–0.5 mg. cell-nitrogen per ml. and took up 5–7 $\times$ 10^3 cmm. H_2/hr./mg. cell-N at high carbon dioxide tensions. If not used immediately the cells were stored at 3°C. *in vacuo;* under these conditions they retain their full activity for at least two days.

All rate measurements were made at 37°C. by the Warburg manometric technique.

The H_2-CO_2 Reaction.—Since our strain of *Mb. omelianskii* has been shown to form methane from hydrogen and carbon dioxide,[6] it is only necessary to present indirect evidence that the same reaction occurs under the conditions of our manometric experiments. For this purpose the ratio H_2 uptake/CO_2 uptake, which according to equation (1) should equal 4, has been determined. A value of 3.53 was found (table 1). In arriving at this value, the hydrogen uptake was calculated from pressure changes on the assumption that one volume of methane was produced for each four volumes of hydrogen disappearing. Carbon dioxide uptake was calculated on the basis of the initial and final bicarbonate and the partition of carbon

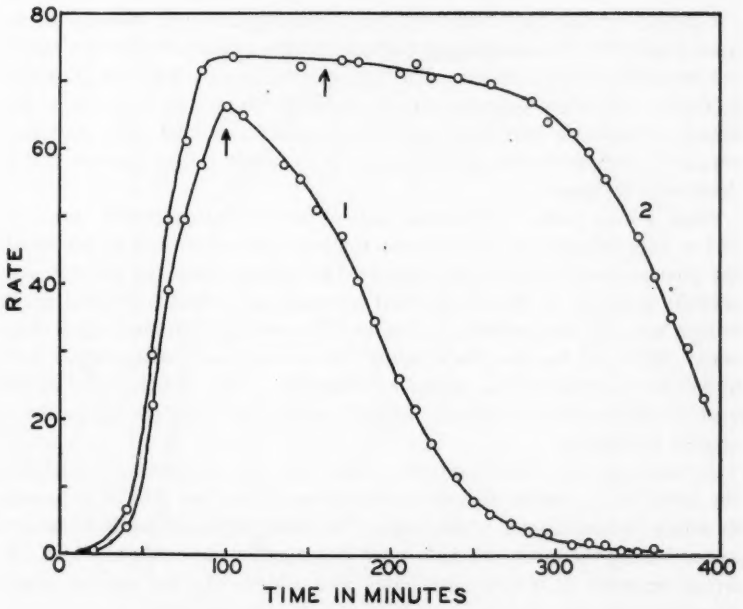

FIGURE 1

Experimental rate-time curves from which the rate-carbon dioxide concentration curve of figure 2 is derived. Each vessel contained 0.2 ml. of a bacterial suspension containing 0.069 mg. cell-N, 0.4-ml. *M*/2 phosphate buffer, pH 6.64, 0.3 ml. *M*/25 (Curve 1) or *M*/10 (Curve 2) bicarbonate-carbonate solution. Total volume, 2 ml. Gas phase, oxygen-free hydrogen, 37°C. The rate is expressed in arbitrary units.

dioxide between the liquid and gas phases. In control experiments the carbon dioxide production in the absence of hydrogen (N_2 atmosphere) was negligible and the rate of hydrogen uptake by cells suspended in a

TABLE 1

THE H_2-CO_2 REACTION

	CMM.
Initial CO_2	166.3
Final CO_2	73.9
CO_2 uptake	92.4
H_2 uptake	326
H_2/CO_2	3.53

phosphate buffer was less than 10 per cent of that by cells in a bicarbonate buffer. In view of several approximations involved in calculating the hydrogen-carbon dioxide ratio by the above method, the agreement between the experimental value and equation (1) may be considered satisfactory.

Changes in Rate with Time.—When a cell suspension is allowed to act upon a mixture of hydrogen and carbon dioxide, a constant reaction rate is not immediately established. The rate is initially very low, but gradually increases until after a period varying from 30 minutes to as much as five hours, it reaches a maximum level which persists as long as an adequate supply of carbon dioxide and hydrogen is available. This phenomenon is illustrated in figure 1.

Since a long period of changing rate is obviously undesirable in manometric experiments, an attempt was made to find conditions under which the phenomenon could be eliminated. This attempt was not entirely successful; however, it was found that the maximum rate is reached much sooner with cell suspensions not more than one day old than with older cells. With old but not with young cells a two-hour pretreatment with hydrogen shortens the lag period considerably. The addition of 0.03 per cent $Na_2S \cdot 9H_2O$ has no effect. In acid media (pH < 6) the lag period is greatly prolonged.

Influence of CO_2 Concentration.—There are two methods for studying the influence of carbon dioxide concentration upon the rate of a process in which carbon dioxide is used up. The multiple vessel method consists in comparing the initial reaction rates in a number of vessels to which different amounts of bicarbonate have been added. In the second, single vessel method, the decline in reaction rate is followed as carbon dioxide is used up. Preliminary experiments showed that the multiple vessel method cannot readily be applied with *Mb. omelianskii* because of the long lag period during which the initial carbon dioxide concentration is greatly reduced. If the initial concentration is small a constant rate is never attained. The single vessel method was therefore used for the final experiments. The well-known objection to this method, namely, that the accumulation of reaction products retards the rate, appears to be unimportant in the present instance.

In applying the single vessel method, a strongly buffered medium was used so the pH and consequently the ratio of carbonic acid to total carbonate would not change appreciably during the experiment. A pH 6.6 phosphate buffer was chosen in order that the rate would be nearly maximal and independent of pH (Fig. 3). The actual pH after addition of the bicarbonate-carbonate mixture was about 6.8. A hydrogen atmosphere was used. After temperature equilibration the bicarbonate was mixed with the cell suspension and the pressure decrease was followed for several hours until exhaustion of carbon dioxide reduced the rate practically to zero. The concentration of carbon dioxide at any given time was calculated from the initial and final levels by making the entirely justifiable assumption that the disappearance of carbon dioxide was proportional to the pressure change. In control experiments, the decline in bicarbonate was measured directly by adding excess acid after various intervals.

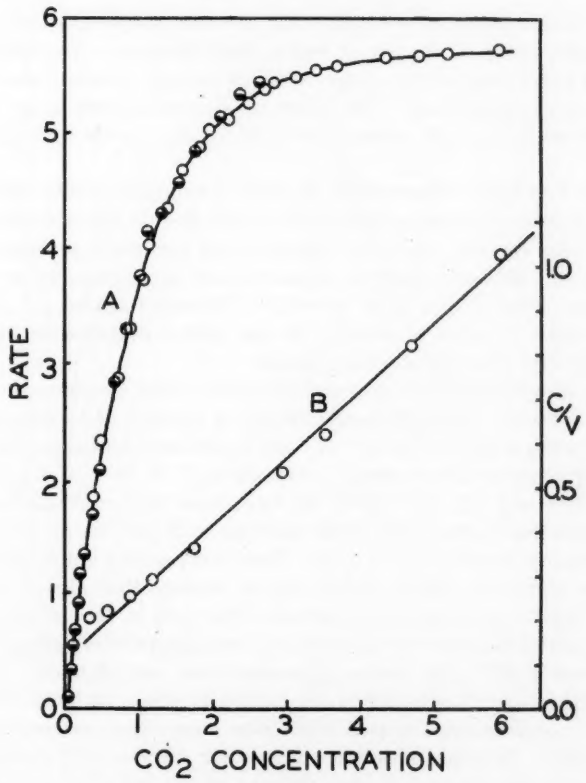

FIGURE 2

The influence of carbon dioxide concentration on the rate of hydrogen uptake (Curve *A*). The rate of hydrogen uptake is expressed in cmm. H_2/hr./mg. cell-N $\times 10^3$. Carbon dioxide concentration is expressed in moles/liter $\times 10^{-4}$. Curve *B:* The ordinate (right) is C/V, where C is carbon dioxide concentration and V is the rate of hydrogen uptake.

The primary data of a typical experiment are presented graphically in figure 1 and the derived carbon dioxide concentration-rate curve is given in figure 2, Curve *A*. In this experiment two vessels were used, one with 0.3 ml. $M/25$, the other with 0.3 ml. $M/10$ bicarbonate. With both vessels, about 100 minutes elapsed before a maximum reaction rate was reached. In vessel No. 1, with the smaller amount of bicarbonate, the rate immediately began to decline as a result of the decrease in carbon dioxide concentration. In vessel No. 2 with more bicarbonate, the rate re-

mained almost constant for more than an hour following the maximum since carbon dioxide was not at first a limiting factor. The two vessels together cover most of the range in which carbon dioxide concentration controls the reaction rate. The points on Curve *A*, figure 2, are derived from the portions of the smooth curves of figure 1 to the right of the arrows.

Curve *A* of figure 2 shows that the rates of hydrogen uptake and carbon dioxide reduction change greatly with carbon dioxide concentration. The shape of the curve is similar to that for other enzymatic processes. The data fit the Michaelis-Menton equation only approximately as can be seen from Curve *B* (Fig. 2) in which C/V is plotted against C.[7] The departure from linearity is greatest at low carbon dioxide concentrations where the experimental errors are largest.

The position of the rate-concentration curve on the concentration axis is of some interest. The half maximum rate is attained at a carbon dioxide concentration of about 7×10^{-5} *M*. With photosynthetic algae, the corresponding concentration is much lower, about 7×10^{-6} *M* for *Chlorella pyrenoidosa*[5] and 1×10^{-6} *M* for the fresh water diatom *Nitzschia palea*.[1] Half saturation of the formic hydrogenlyase of *B. coli*[8] occurs at a higher concentration, about 1.5×10^{-3} *M*. These comparisons indicate that bacteria are adapted to higher carbon dioxide tensions than algae. This difference can be correlated with normal differences in the carbon dioxide content of the environments in which the two groups of organisms live.

Influence of pH.—The above experiment does not allow one to decide whether the rate is determined by free carbon dioxide or by bicarbonate ion, since the concentrations of both substances vary simultaneously and proportionately. In order to decide which factor is the more important, the reaction rate was determined when the ratio of carbonic acid to bicarbonate was varied by changing the pH. Two types of pH-rate experiments were carried out. In one, the carbon dioxide concentration was kept constant by equilibrating the medium with a mixture of 8.75 per cent carbon dioxide in hydrogen. At this concentration (2.2×10^{-3} *M*), carbon dioxide does not limit the rate. The pH was controlled by varying the bicarbonate concentration. The results of two experiments are given in table 2. In the second type of pH-rate experiment the total carbonate concentration was kept constant while the pH, carbon dioxide and bicarbonate ion concentrations were varied. The maximum rates of hydrogen uptake were determined in a medium containing 0.1 *M* phosphate or phosphate-acetate buffer and 0.010–0.015 *M* $NaHCO_3$. The quantity of bicarbonate was large enough so that the carbon dioxide concentration would not decrease greatly in short experiments and was small enough to avoid any considerable change in pH of the buffer. The pH was determined with a glass electrode at the end of each experiment. The results of five experiments are summarized in figure 3.

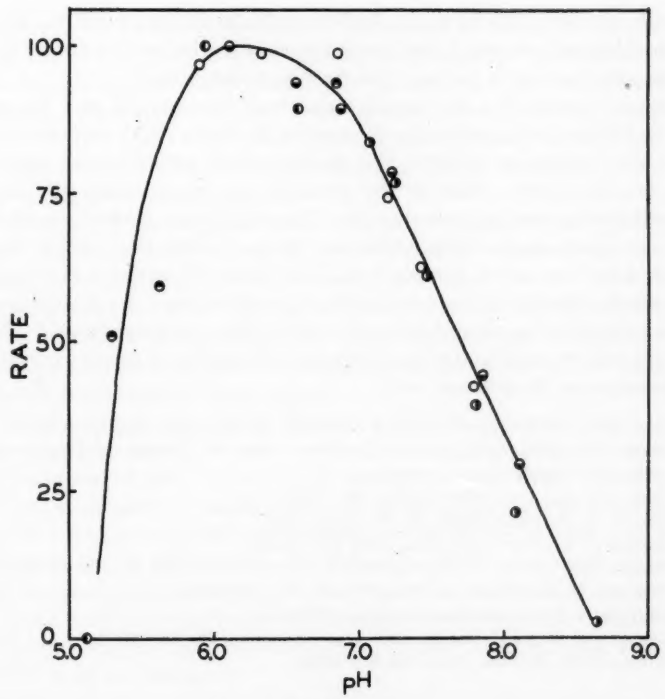

FIGURE 3

The influence of pH on the rate of hydrogen uptake, expressed in arbitrary units, when the total carbonate concentration is low (0.010–0.015 *M*) and constant.

TABLE 2

INFLUENCE OF pH ON THE RATE OF CARBON DIOXIDE REDUCTION AT A HIGH CO_2 TENSION (Gas phase: 8.75 per cent CO_2 in H_2)

APPROX. HCO_3^- CONC.	pH	RELATIVE RATE
0.000	5.80	81
0.002	6.57	82
0.008	6.94	87
0.012	7.11	82
0.03	7.42	82
0.06	7.72	100
0.10	8.00	74
0.20	8.19	57

The data of table 2 show that when the partial pressure of carbon dioxide is kept constant, the rate is nearly independent of pH and bicarbonate concentration over the range from pH 5.8 to 7.4. At pH 7.7 the rate is

slightly greater while in more alkaline media it declines rapidly. On the basis of this experiment it is impossible to decide whether the decline is due to the alkalinity or to the high bicarbonate concentration.

Figure 3 shows that the rate declines with increasing pH over the range 6.4 to 8.6 in which carbon dioxide tension decreases and bicarbonate concentration increases. Since in this experiment the rate decreases with carbon dioxide tension, while in the previous experiment where the carbon dioxide tension was kept constant the rate was largely independent of both pH and bicarbonate concentration, one must conclude that carbon dioxide is the substance which combines with the catalytic system and primarily determines the rate at least over the pH range 6.0 to 7.8. A similar conclusion has been reached in the case of green plant photosynthesis.[5] Above pH 7.8 and below pH 6 the rate is determined mainly by the concentrations of hydrogen or bicarbonate ions.

* The ability of the organism to use molecular hydrogen was discovered about 1940 by Charles Schnellen, working in the laboratory of Prof. A. J. Kluyver. Because of the war, Schnellen's thesis is not yet available.

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EFFECT OF COLCHICINE PRETREATMENT ON THE FREQUENCY OF CHROMOSOMAL ABERRATIONS INDUCED BY X-RADIATION

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Recent investigations indicate that the frequency of chromosomal aberrations induced by x-radiation is influenced by the amount of chromosomal movement during radiation. Increased chromosomal movement results in a higher frequency of aberrations since there is a greater opportunity for broken ends to fuse in new combinations. Thus centrifuging the cells during radiation tends to increase the frequency of induced aberrations (Sax[1]). Prophase stages are more sensitive than resting stages partly because of greater movement in the prophasic nucleus. The differential

sensitivity of different types of cells and tissues to x-rays is apparently associated with differences in the amount of chromosomal movement at the time of radiation (Sax and Swanson[2]). Radiation at high intensity results in a greater aberration frequency than the same dose given at a low intensity, indicating that the frequency of illegitimate fusions depends on the time interval that the breaks remain "open" as well as the amount of movement during this interval (Sax[3]).

Colchicine inhibits anaphase movement of chromosomes and apparently retards other stages of the mitotic cycle (Eigsti[4]). Thus the use of colchicine offers another means whereby the relationship between the amount of chromosomal movement and the frequency of x-ray induced aberrations can be tested. The results presented here indicate that treatment with colchicine prior to radiation reduces the frequency of aberrations induced by x-radiation at the prophase stages.

Methods.—Onion bulbs of the White Marglobe variety were used. Four bulbs were germinated in water until a number of roots 2.0–2.5 cm. in length were developed. Two of them were placed with their roots dipping into a 0.05% solution of colchicine for 45 minutes, the other two serving as controls. The roots of the control bulbs and colchicine treated ones were then subjected to 300 r units of x-radiation, the x-ray treatment being given both lots simultaneously. Following radiation both lots were placed with their roots in a 0.05% solution of colchicine for 48 hours. At the end of this time the root tips were fixed in 3:1 alcohol-acetic, stained with the Feulgen stain and smeared.

Treatment with colchicine following radiation inhibits anaphase, thus insuring that none of the aberrations is lost by cell division subsequent to radiation and at the same time increases the number of metaphases available for study. Some tetraploid cells were found after this treatment, however, this report is limited to the effects observed in diploid cells of the pretreated roots and controls. A comparison of the types and frequencies of breaks in tetraploid and diploid cells will appear in a separate report (Sax and Brumfield[5]).

Experimental Results.—The types of aberrations observed in both the experimental and control roots were the same as those reported by Sax for x-irradiated *Allium* roots (Sax[6]) and *Tradescantia* microspores (Sax[7]). However, because of the use of the colchicine after radiation, secondary rings and dicentrics did not occur. The results are presented in table 1 which shows the frequencies, expressed as number of aberrations per 100 chromosomes, of different types of induced aberrations. As seen from the table, total aberration frequency in the controls was about twice that of the colchicine pretreated material. Chromosome effects were found with about the same frequency in both lots. Significant differences ($p < 0.01$) were found in the frequency of chromatid effects, both the one-hit and two-

hit aberrations being about 3.5 times as frequent in the controls as in the colchicine pretreated roots.

Discussion.—Chromatid effects are induced in cells irradiated during prophase stages when the chromosomes are effectively split while chromosome aberrations result from radiation of resting nuclei, the chromosome then being effectively single. Thus those cells having chromatid effects were evidently in prophase at the time of the colchicine pretreatment and x-radiation while those showing chromosome effects were in resting stages. The results presented here indicate that the colchicine pretreatment did not appreciably affect the frequency of aberrations induced in resting nuclei, but significantly reduced the frequency of aberrations induced in prophase stages. Chromosome movement is presumably at a minimum during the resting stage. Accordingly, colchicine pretreatment, by reducing chromosome movement, should have little differential effect on the frequency of chromosome aberrations induced in the pretreated roots as compared with the controls. During prophase, however, with the development of new minor coils and reduction in the number of relational coils, there is considerable movement of the chromosomes. This gives a greater opportunity for broken ends to fuse in new combinations and is partly responsible for the sensitivity of prophase nuclei being greater than that of resting nuclei. Slower movement would evidently result in reduced sensitivity to x-rays and this is most probably the mechanism whereby colchicine pretreatment affects a lower frequency of chromatid effects induced by x-radiation.

Guyer and Clark,[8] found that cancer tissue pretreated with colchicine for 15 hours was more sensitive to x-radiation than untreated controls, and Levine[9] reported that onion roots treated with a 0.01% colchicine solution for 48 hours were more sensitive than untreated roots. In these cases, however, the colchicine pretreatment was of sufficient duration to cause a considerable increase in the number of metaphases present, a stage of high sensitivity to x-radiation. Consequently these results are not directly comparable to the experiment presented here, since in this case, the 45-minute colchicine pretreatment was not sufficient to appreciably increase the number of metaphase stages (cf. Levine and Gelber[10]).

Summary.—Treatment of onion roots for 45 minutes with a 0.05% solution of colchicine prior to x-radiation resulted in less than one-third as many chromatid aberrations as found in non-colchicine treated controls. The frequency of x-ray induced chromosome effects was about the same in pretreated roots and controls. The colchicine pretreatment presumably reduces the amount of chromosome movement in prophase where chromatid effects are induced, thus resulting in less opportunity for fusion of broken ends into new combinations.

TABLE 1

SHOWING THE FREQUENCY OF ABERRATIONS, EXPRESSED AS NUMBER OF ABERRATIONS PER 100 CHROMOSOMES, INDUCED BY X-RADIATION IN ONION ROOTS TREATED WITH A 0.05% COLCHICINE SOLUTION FOR 45 MINUTES PRIOR TO X-RADIATION, AND CONTROLS. 300 R

	TOTAL CHROMOSOMES	CHROMATID ABERRATIONS			CHROMOSOME ABERRATIONS			TOTAL ABERRATIONS
		ONE-HIT	TWO-HIT	TOTAL	ONE-HIT	TWO-HIT	TOTAL	
Colchicine pretreated	4448	0.79	0.24	1.03	0.94	0.81	1.75	2.78
Controls	6272	2.66	0.85	3.51	0.65	1.18	1.83	5.34

This experiment was carried out at Harvard University while the author was holder of a National Research Fellowship. The author wishes to express his sincere appreciation to Prof. Karl Sax for suggestions and criticisms.

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SOME TOPOLOGICALLY INVARIANT METRIC PROPERTIES

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According to the well-known duality theorem of Alexander, each simple closed curve C in cartesian n-space E_n is linked by an $n-2$ cycle Z in $E_n - C$. In the sequel it will be shown that Z can frequently be taken to be a metric $n-2$ dimensional sphere. A generalization will be formulated which holds not only in E_n, but in an arbitrary metric space. As an application, solutions will be given to two hitherto unsolved problems proposed, respectively, by L. M. Blumenthal and I. J. Schoenberg.

Let p and q be two points of a metric space M; $D(p, q)$ the set of points x such that $px = xq \leqq pq$; $S(p, q)$ the set of points x such that $px = xq = pq$; $H(p, q)$ the set of points x such that $px = xq$. $D(p, q)$, $S(p, q)$ and

$H(p, q)$ will be called, respectively, the disc, the sphere, and the subspace dual to p and q in M. $H(p, q)$ has two sides; the p-side consisting of the points of M whose distances from p are less than their distances from q, and a q-side similarly defined.

An arc A in M will be said to be in general position relative to a disc $D(p, q)$ if neither end-point of A is in $D(p, q)$ and A does not intersect $S(p, q)$. If A is in general position relative to a disc $D(p, q)$, then, in close analogy to the procedure for cartesian E_n, it is possible to define an intersection number $I(A;\ p, q)$ between A and $D(p, q)$. Taken mod 2, $I(A;\ p, q)$ will be either 0 or 1. For any arc A' sufficiently close to A, and points p', q' sufficiently near p and q, we have $I(A;\ p, q) = I(A';\ p', q')$. For any sum of arcs ΣA, each in general position relative to $D(p, q)$, we have $I(\Sigma A;\ p, q) = \Sigma I(A;\ p, q)$.

We define $I(A;\ p, q) = 1$ if the end-points of A are on opposite sides of $H(p, q)$ and A does not intersect $H(p, q)$–$D(p, q)$. We define $I(A;\ p, q) = 0$ if A does not intersect $D(p, q)$, or if both end-points of A are on the same side of $H(p, q)$ and A does not intersect $H(p, q)$–$D(p, q)$. Each arc in general position relative to the disc $D(p, q)$ can be expressed as a finite sum of arcs of these three types, $A = \Sigma A'$, and $I(A;\ p, q) = \Sigma I(A';\ p, q)$, mod 2. It can be shown that $I(A;\ p, q)$ is independent of the way A is decomposed into the sum $\Sigma A'$.

We may now state our principal theorem.

INTERSECTION THEOREM: *Let A be an arc with end-points a and b contained in the metric space M. If S is a segment in A, then*

1. S contains two points p and q such that $D(p, q)$ does not intersect the subarcs (ap) and (qb); distance $[(ap), (qb)] = pq$;

2. If S contains no equilateral triple, i.e., three points p_1, p_2 and p_3 such that $p_1p_2 = p_2p_3 = p_3p_1$, then for any such p and q we have $I(A;\ p, q) = 1$.

COROLLARY 1. *If a simple closed curve C contains one subsegment S without an equilateral triple, then S contains two points p and q such that $I(C;\ p, q) = 1$.*

This corollary can be interpreted as a linking theorem since, in E_n, $S(p, q)$ will be an n–2 dimensional metric sphere, and will link C.

COROLLARY 2. *If in E_n a simple closed curve C possesses a tangent at a point p, then each segment containing p contains two points whose dual sphere links C.*

LEMMA 1. *Each simple closed curve C in any metric contains an equilateral triangle.*[1]

Assume the contrary. Then $I(C;\ x, y)$ is defined for any x, y in C and continuous in x, y. Being an integer it is constant. But if p' and q' are two points of C whose distance is maximal then clearly $I(C;\ p', q') = 0$, but corollary 1, together with this, yields a contradiction.

LEMMA 2. *If T is a tripod, that is, the sum of three free arcs having one*

and the same end-point in common, then, in each metric, T contains an equilateral triangle.

Call A the sum of two of the three arcs. Assuming the Lemma false, $I(A;\ x, y)$ is defined for each x, y in T. It is continuous, hence is readily seen to be constant. For two points p, q close together on this third arc but not near A, we have $I(A;\ p, q) = 0$. The Intersection Theorem applied to A combines with this observation to yield a contradiction. These two lemmas combine to yield

THEOREM 2. *Each continuous curve which is not a simple arc contains in each metric an equilateral triple.*

A sequence of n points $p_1, p_2, \ldots, p_n$ contained in an arc A and arranged in natural order will be called a polygon on A. Similarly we may have a polygon on a simple closed curve. If $p_1 = p_n$ the polygon is called closed, and is called equilateral if $p_1p_2 = p_2p_3 = \ldots = p_{n-1}p_n$.

The n-Lattice Theorem, due to Menger, states that on any arc, for each integer n, and in each metric, there exists an equilateral polygon of n sides. Menger's proof of this theorem was incomplete. Alt and Beer gave a proof for arcs contained in E_n. Later, I. J. Schoenberg gave a proof of the theorem for general arcs. Schoenberg raised the question as to whether the theorem is true for closed polygons on closed curves. As an application of our Intersecton Theorem, together with the well known theory of the degree of a mapping of an $n-1$ sphere into E_n we can prove

THE CLOSED N-LATTICE THEOREM. *A simple closed curve contains, in each metric and for each positive integer n, a closed equilateral polygon of n sides.*

In conclusion we should like to mention the following open problem: Does the conclusion of Corollary 1 hold without assumption of the existence of no equilateral tripleon S?

[1] Lemmas 1 and 2, together with Theorem 2, were conjectured by L. M. Blumenthal. The author presented a proof of these to the American Mathematical Society at the November meeting, 1942.

ON A STATISTICAL GENERALIZATION OF METRIC SPACES

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In a very interesting paper Karl Menger[1] has recently introduced a statistical generalization of semi-metric and metric spaces. According to Menger a set R of elements (points) is called a statistical semi-metric space if with each pair of points p and q of the space R a real function $F(x;\ p, q)$ is associated satisfying the following conditions:

1. $F(x;\ p, q) = 0$ for $x \leq 0$ and $\lim_{x=\infty} F(x;\ p, q) = 1$.
2. $F(x;\ p, q)$ is a non-decreasing function of x and continuous to the left.
3. $F(x;\ p, q) = F(x;\ q, p)$ for any pair of points p and q.
4. $F(x;\ p, p) = 1$ for any $x > 0$.

The function $F(x;\ p, q)$ can be interpreted as the probability distribution function of the distance of p and q; i.e., for any value x, $F(x;\ p, q)$ denotes the probability that the distance of p and q is less than x. In all that follows a distribution function will mean a function of a real variable x which satisfies conditions 1 and 2.

As a statistical generalization of the triangular inequality in metric spaces the following inequality has been proposed by Menger: For any three points p, q and r we have

5. $T[F(x;\ p, q),\quad F(y;\ q, r)] \leq F(x + y;\ p, r)$

where $T(a, b)$ is a function of two variables satisfying certain conditions. A statistical semi-metric space is called a statistical metric space if inequality 5 is satisfied for all triples, p, q and r.

Menger's generalization of the triangular inequality has the drawback that it involves an unspecified function $T(a, b)$ and one can hardly find sufficient justification for a particular choice of this function. Furthermore the notion of "between" introduced by Menger on the basis of inequality 5 has the properties of the between relationship in metric spaces only under restrictive conditions on the distribution functions $F(x;\ p, q)$. Here we propose another statistical generalization of the triangular inequality which is free from the above mentioned difficulties.

By the symbolic sum $F(x) \overset{\circ}{+} G(x)$ of two distribution functions $F(x)$ and $G(x)$ we mean the distribution function $H(x)$ given by the Stieltjes integral

$$H(x) = \int\int d[F(u)G(v)]$$

where the integration is to be taken over the domain of the (u, v) plane

given by the inequality $u + v < x$. Thus if X and Y are independently distributed random variables with the distribution functions $F(x)$ and $G(x)$, respectively, then $F(x) \overset{\circ}{+} G(x)$ is the distribution function of $X + Y$.

In all that follows for any two distribution functions F and G the symbol $F \leq G$ will mean that the inequality $F(x) \leq G(x)$ holds for all values of x. The symbol $F < G$ will mean that $F \leq G$ and F is not identically equal to G. The symbols $F \geq G$ and $F > G$ are synonymous with the symbols $G \leq F$ and $G < F$, respectively.

As a statistical generalization of the triangular inequality we propose the following inequality: For any three points p, q, r we have

$$5'. \quad F(x;\ p, q) \overset{\circ}{+} F(x;\ q, r) \leq F(x;\ p, r).$$

We will say that two points p and q are different, in symbol $p \neq q$, if there exists a positive value x such that $F(x;\ p, q) < 1$. We will say that a point q lies between the points p and r if

$$6. \quad p \neq q \neq r \neq p,$$

and

$$7. \quad F(x;\ p, q) \overset{\circ}{+} F(x;\ q, r) = F(x;\ p, r).$$

Let the symbol pqr denote the relationship that q lies between p and r. It can be shown that the "between" relationship defined here has the same properties as the between relationship in metric spaces,[2] i.e., our statistical between relationship satisfies the following conditions:

I. From pqr it follows that rqp.
II. From pqr it follows that qrp and rpq cannot hold.
III. From pqr and prs it follows that pqs and qrs.

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A SUFFICIENT CONDITION THAT A C-CHARACTERISTIC BE GEOMETRIC

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Introduction.—If $\{\gamma\} = \{\gamma_0; \gamma_1, \gamma_2, \ldots, \gamma_\rho\}$ is the characteristic of a homaloidal net defined at a set of ρ points, then $\{\gamma\}$ must satisfy Cremona's equations

$$\begin{aligned} x_1^2 + x_2^2 + \ldots + x_\rho^2 - x_0^2 &= -1 \\ x_1 + x_2 + \ldots + x_\rho - 3x_0 &= -3. \end{aligned} \tag{1}$$

Further, if the multiplicities γ_i are ordered so that $\gamma_1 \geqq \gamma_2 \geqq \gamma_3 \geqq \ldots \geqq \gamma_\rho$, $\{\gamma\}$ must satisfy the set of inequalities

$$\begin{aligned} \gamma_0 &\geqq \gamma_1 + \gamma_2 \\ 2\gamma_0 &\geqq \gamma_1 + \gamma_2 + \ldots + \gamma_6 \\ 3\gamma_0 &\geqq 2\gamma_1 + \gamma_2 + \ldots + \gamma_7 \\ 3\gamma_0 &\geqq \gamma_1 + \gamma_2 + \ldots + \gamma_9 \\ &\ldots\ldots\ldots\ldots\ldots\ldots \end{aligned} \tag{2}$$

These inequalities express the fact that no curve of order k and defined at the ρ points can meet the net in more than $\gamma_0 k$ points. Coble[1] has conjectured that the conditions (1) and (2) are sufficient as well as necessary.

In this paper we will show that the conditions (2) are actually sufficient. Indeed, the less stringent set of inequalities given in (5) will suffice. The notation and point of view is that used in "Cremona's Diophantine Equations."[2]

We begin with the proof of

LEMMA: *If $c_0 > 0$ in a solution $\{c\}$ of equations (1), then $2c_0 > c_1 + c_2 + c_3$, where*

$$c_1 \geq c_2 \geq c_3 \geq \ldots \geq c_\rho \tag{3}$$

It is clear that $c_0 > c_1 \geq c_2 \geq c_3$. Let us write

$$c_1 = c_0 - a_1; \quad c_2 = c_0 - a_2; \quad c_3 = c_0 - a_3;$$

where a_1, a_2, a_3 are all positive. Substitution in (1) yields

$$\begin{aligned} c_4^2 + \ldots + c_\rho^2 &= 2(a_1 + a_2 + a_3)c_0 - 2c_0^2 - (a_1^2 + a_2^2 + a_3^2) - 1 \\ c_4 + \ldots + c_\rho &= (a_1 + a_2 + a_3) - 3. \end{aligned}$$

If $a_1 + a_2 + a_3 - 3 = 0$, then $a_1 = a_2 = a_3 = 1$ and it is easily verified that in this case $\{c\} = \{1; 0^\rho\}$ or $\{2; 1^3 0^{\rho-3}\}$. Both of these satisfy the conditions of the lemma.

If $a_1 + a_2 + a_3 - 3 > 0$, it follows that $c_4 + \ldots + c_\rho > 0$ and $c_4^2 + \ldots + c_\rho^2 > 0$. Then,

$$\text{or} \qquad \begin{aligned} 2(a_1 + a_2 + a_3)c_0 &> 2c_0^2 + (a_1^2 + a_2^2 + a_3^2) + 1 \\ 2(a_1 + a_1 + a_2)c_0 &> 2c_0^2, \end{aligned}$$

whence, since $c_0 > 0$

$$a_1 + a_2 + a_3 > c_0.$$

Then

$$2c_0 > (c_0 - a_1) + (c_0 - a_2) + (c_0 - a_3) = c_1 + c_2 + c_3.$$

From this inequality we readily deduce

If a solution $\{c\}$ of (1) has $c_0 > 0$, then the conjugate $\{c'\}$ of $\{c\}$ under A_{123} has $c_0' > 0$. (4)

A_{123} has the equations $x_0' = 2x_0 - x_1 - x_2 - x_3$; $x_i' = x_i + (x_0 - x_1 - x_2 - x_3)$, $i = 1, 2, 3$. Thus $c_0' = 2c_0 - c_1 - c_2 - c_3$ and is, in accordance with (3), always positive. This means that the property $c_0 > 0$ of a solution of (1) is invariant under the group generated by A_{123} and the permutation group $\pi(x_1, x_2, \ldots, x_\rho)$. We are now in a position to demonstrate:

Let $\{\gamma\}$ be a solution of equations (1), arranged so that $\gamma_1 \geq \gamma_2 \geq \ldots \geq \gamma_\rho$. Further, let $\{\gamma\}$ satisfy the set of inequalities

$$p_0\gamma_0 \geq p_1\gamma_1 + p_2\gamma_2 + \ldots + p_\rho\gamma_\rho,$$

where the characteristics $\{p\}$ run over the finite set of all geometric P-characteristics with $p_0 > \gamma_0$ and are ordered so that $p_1 \geq p_2 \geq \ldots \geq p_\rho$. Then $\{\gamma\}$ is geometric. (5)

We remark that $\{0;\ 0^{\rho-1}, -1\}$ is a geometric P-characteristic so that $\gamma_\rho > 0$. Also $\gamma_0 \geq \gamma_1 + \gamma_2$ and $\gamma_0 > 0$. Since all the multiplicities γ_i are positive, it follows that $\gamma_0 < \gamma_1 + \gamma_2 + \gamma_3$. Thus under A_{123}, $\{\gamma\} \to \{\gamma'\}$, where $\gamma_0 > \gamma_0^1 > 0$. This process of reduction may be continued until one of two things happens. $\{\gamma\}$ may be reducible under A_{123} and π to $\{1;\ 0^\rho\}$, in which case $\{\gamma\}$ is geometric. On the other hand, after a certain number of reductions, an irreducible characteristic $\{\bar{\gamma}\}$ may be obtained. In accordance with (3), $\bar{\gamma}_0 > 0$. Since $\{\bar{\gamma}\}$ is irreducible, it follows that at least one $\bar{\gamma}_i$, say $\bar{\gamma}_\rho$, is negative. Now let T,

$$T: \quad \begin{aligned} x_0' &= nx_0 - r_1x_1 - \ldots - r_\rho x_\rho \\ x_1' &= s_1x_0 - \alpha_{11}x_1 - \ldots - \alpha_{1\rho}x_\rho \\ &\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \\ x_\rho' &= s_\rho x_0 - \alpha_{\rho 1}x_1 - \ldots - \alpha_{\rho\rho}x_\rho, \end{aligned} \qquad (6)$$

be the transformation which sends $\{\gamma\} \to \{\bar{\gamma}\}$. Then

$$\bar{\gamma}_\rho = s_\rho\gamma_0 - \alpha_{\rho 1}\gamma_1 - \ldots - \alpha_{\rho\rho}\gamma_\rho.$$

Since $\bar{\gamma}_\rho < 0$, we have

$$s_\rho\gamma_0 < \alpha_{\rho 1}\gamma_1 + \ldots + \alpha_{\rho\rho}\gamma_\rho.$$

That is, the P-characteristic $\{s_\rho;\ \alpha_{\rho 1}, \ldots, \alpha_{\rho\rho}\}$ does not satisfy the condition of the theorem. If $s_\rho < \gamma_0$, we will have a contradiction; i.e., an irreducible characteristic cannot arise under the conditions given in (5).

To prove that $s_\rho < \gamma_0$, consider T^{-1}, the inverse of T.

$$T^{-1}: \begin{aligned} x_0' &= nx_0 - s_1x_1 - \ldots - s_\rho x_\rho \\ x_1' &= s_1x_0 - \alpha_{11}x_1 - \ldots - \alpha_{\rho 1}x_\rho \\ &\ldots\ldots\ldots\ldots\ldots\ldots\ldots \\ x_\rho' &= s_\rho x_0 - \alpha_{1\rho}x_1 - \ldots - \alpha_{\rho\rho}x_\rho. \end{aligned} \qquad (7)$$

Write $\bar{\gamma} = \{\bar{\gamma}_0, \bar{\gamma}_1, \bar{\gamma}_2, \ldots, \bar{\gamma}_{\rho-1}, 0\} + k\{0;\ 0^{\rho-1} - 1\}$, $k > 0$. Under T^{-1}, $\{\bar{\gamma}_0;\ \bar{\gamma}_1, \bar{\gamma}_2, \ldots, \bar{\gamma}_{\rho-1}, 0\} \rightarrow \{d_0;\ d_1, d_2, \ldots, d_\rho\}$ with $d_0 > 0$. For otherwise, the net defined at $\rho + 1$ points $\{n;\ s_1, s_2, \ldots, s_{\rho-1}, 0, s_\rho\}$ would yield a T' which sends $\{\bar{\gamma}\}$ into a $\{\bar{\gamma}'\}$ with $\bar{\gamma}_0' \leq 0$, which is impossible, according to (3). Under T^{-1}, $k\{0;\ 0^{\rho-1}-1\} \rightarrow k\{s_\rho;\ \alpha_{\rho 1}, \ldots, \alpha_{\rho\rho}\}$. But since $\{\bar{\gamma}\} \rightarrow \{\gamma\}$, we have $\{\gamma\} = \{d_0;\ d_i\} + k\{s_\rho;\ \alpha_{\rho i}\}$, and

$$\gamma_0 = d_0 + ks_\rho.$$

Since d_0, k are positive, it follows that $s_\rho < \gamma_0$ the contradiction is complete, and (5) is established.

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